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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/57, 1/21, 9/56, 15/75, 15/62 — A2

(11) International Publication Number: WO 97/39130

(43) International Publication Date: 23 October 1997 (23.10.97)

(21) International Application Number: PCT/US97/06477

(22) International Filing Date: 16 April 1997 (16.04.97)

(30) Priority Data:
08/634,440 18 April 1996 (18.04.96) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/634,440 (CON)
Filed on 18 April 1996 (18.04.96)

(71) Applicants (*for all designated States except US*): NORTH CAROLINA STATE UNIVERSITY [US/US]; 103 Holladay Hall, Campus Box 7003, Raleigh, NC 27695-7003 (US). UNIVERSITY TECHNOLOGIES INTERNATIONAL, INC. [CA/CA]; 609 14th Street, N.W., Calgary, Alberta (CA).

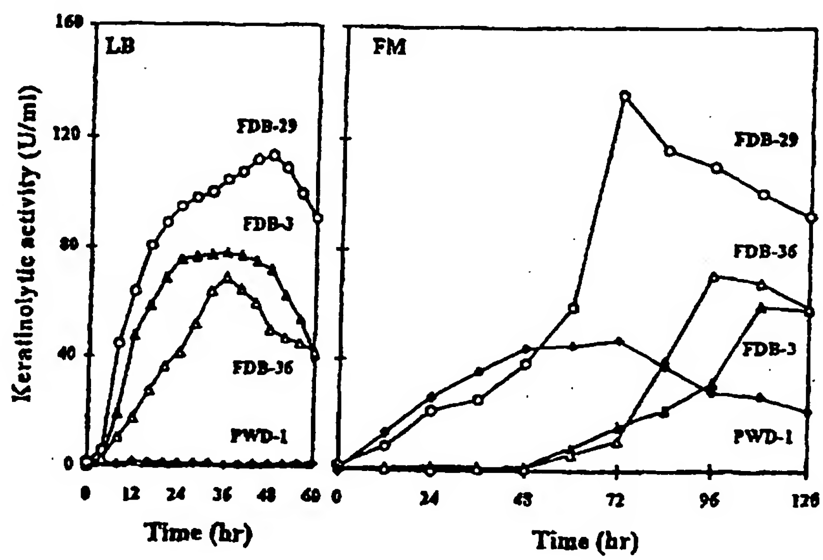
(72) Inventors; and
(75) Inventors/Applicants (*for US only*): SHIH, Jason, C., H. [US/US]; 100 Planters Wood Lane, Cary, NC 27511 (US). LIN, Xiang [CN/US]; Apartment 203, 3608 Horton Street, Raleigh, NC 27607 (US). WONG, Sui-Lam [CA/CA]; 38 Ranch Glen Drive, N.W., Calgary, Alberta T3G 1E3 (CA).

(74) Agents: SIBLEY, Kenneth, D. et al.; Myers, Bigel, Sibley & Sajovec, L.L.P., P.O. Box 37428, Raleigh, NC 27627 (US).

(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: METHOD FOR EXPRESSING AND SECRETING KERATINASE



(57) Abstract

The present invention provides a *Bacillus subtilis* host cell capable of expressing and secreting keratinase. The host cell contains a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith. The present invention also provides a method for producing keratinase enzyme. The method includes the steps of (a) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith, and (b) collecting keratinase enzyme from the cell culture.

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METHOD FOR EXPRESSING AND SECRETING KERATINASE

This invention was made with Government support under grant number NRI-93-37500-9247 from the United States Department of Agriculture. The government has certain rights to this invention.

Field of the Invention

The present invention relates to cloning and expression of enzymes in and secretion by host cells, and in particular to cloning, expression, and secretion of keratinase in host cells.

Background of the Invention

Feathers are produced in large quantities by the poultry industry. These feathers provide an inexpensive source of raw material for a variety of potential uses. Among other things, there has been considerable interest in developing methods of degrading feathers so they can be used as an inexpensive source of amino acids and digestible protein in animal feed. Processes for converting feather into animal feed which have been developed to date include both steam hydrolysis processes and combined steam hydrolysis and enzymatic processes. See, e.g., Papadopoulos, M.C., *Animal Feed Science and Technology* **16**:151 (1986); Papadopoulos, M.C., *Poultry Science* **64**:1729 (1985); Alderibigde, A.O. *et al.*, *J. Animal Science* **1198** (1983); Thomas and Beeson, *J. Animal Science* **45**:819 (1977); Morris *et al.*, *Poultry Science* **52**:858 (1973); Moran *et al.*, *Poultry Science* **46**:456 (1967); Davis *et al.*, Processing of poultry by-products and their utilization in feeds, Part I, USDA Util. Res. Rep. no. 3, Washington, D.C. (1961). Disadvantages of these procedures, such as the

degradation of heat sensitive amino acids by steam processes and the relatively low digestibility of the resulting products, have lead to continued interest in economical new feather degradation procedures which do not require a harsh steam treatment.

Keratinase enzyme has been found to be an effective feather degrading enzyme useful for converting keratin into amino acids for inclusion into animal feeds. U.S. Patent Application Serial No. 08/250,028 filed 27 May 1994 discloses an isolated *Bacillus licheniformis* PWD-1 keratinase enzyme for such use.

It is an object of the present invention to provide new, economical methods of producing keratinase.

It is a further object of the present invention to provide a host cell, and expression and secretion system for keratinase, which is capable of the hyperproduction of keratinase.

It is a further object of the present invention to provide recombinant DNA, host cells, and an expression and secretion system capable of hyperexpressing an enzyme encoded by a heterologous DNA.

Summary of the Invention

The foregoing objects are met by the present invention. As a first aspect, the present invention provides a *Bacillus subtilis* host cell capable of expressing and secreting keratinase. The host cell contains a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith. *Bacillus licheniformis* PWD-1 keratinase enzyme has the sequence as set forth in SEQ ID NO:1. In the preferred embodiment, the vector DNA further comprises a *kerA*

pre/pro processing and secretion region at nucleotides 215 through 529 of the keratinase gene (SEQ ID NO:1).

As a second aspect, the present invention provides a method for producing keratinase enzyme. The method includes the steps of (*a*) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith, and (*b*) collecting keratinase enzyme from the cell culture.

As a third aspect, the present invention provides an expression and secretion system for keratinase enzyme. The expression and secretion system includes (*a*) a *Bacillus subtilis* host cell, and (*b*) a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith.

As a fourth aspect, the present invention provides a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme. The heterologous DNA encoding the enzyme may be a heterologous DNA encoding a proteinase, in particular a keratinase.

As a fifth aspect, the present invention provides a *Bacillus subtilis* host cell capable of expressing and secreting an enzyme encoded by a heterologous DNA. The host cell contains a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme. Preferably, the heterologous DNA is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 keratinase enzyme.

As a sixth aspect, the present invention provides a method of producing an enzyme. The method includes the steps of (*a*) culturing a *Bacillus subtilis*

host cell containing a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme, and (*b*) collecting enzyme from the *Bacillus subtilis* host cell culture. Preferably, the heterologous DNA is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 keratinase enzyme.

As a seventh aspect, the present invention provides an expression and secretion system for an enzyme. The system includes (*a*) a *Bacillus subtilis* host cell, and (*b*) a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme. Preferably, the heterologous DNA is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 keratinase enzyme.

The foregoing and other objects and aspects of the present invention are explained in detail in the detailed description set forth below.

Brief Description of the Drawings

Figure 1 illustrates the construction of a plasmid, pLB3, containing the 1.45 kilobase *kerA* keratinase gene. Km^r denotes the kanamycin resistance gene.

Figure 2 illustrates the structures of plasmids, pLB3, pLB29, and pLB36 all containing the 1.45 kilobase *kerA* keratinase gene. P43 represents the ~300 base pair fragment containing the vegetative growth promoter. Km^r denotes the kanamycin resistance gene. Arrows indicate the orientations of genes.

Figure 3 illustrates the detection of proteolytic activity by formation of hydrolysis haloes on milk-agar plates. Plate A represents cell-free culture supernatants from 72-hour feather medium. Plate B represents 36-hour cell-free culture supernatants from Luria-Bertani medium. The numbers on

the plates represent culture supernatants from (1) PWD-1, (2) FDB-3, (3) FDB-29, (4) FDB-36, and (5) DB104/PUB18.

Figure 4 is a graphical illustration of the expression of *kerA* in FDB-3, FDB-29, and FDB-36 in Luria-Bertani (LB) medium and feather medium (FM). Keratinolytic activity was measured by azokeratin hydrolysis.

Figure 5 illustrates the immuno-diffusion assay of keratinase produced in culture media using rabbit anti-keratinase serum. Plate A contains cell-free culture supernatnats from feather medium. *Bacillus licheniformis* PWD-1 and FDB-29 sampels were taken at 72 hours, FDB-3, FDB-36, and DB104/pUB18 samples were taken at 96 hours. Plate B contains 36-hour cell-free culture supernatants from LB medium. The numbers on plates represent culture supernatants from (1) PWD-1, (2) FDB-3, (3) FDB-29, (4) FDB-36, and (5) DB104/PUB18.

Figures 6A, 6B, and **6C** are graphical illustrations of the effects of kanamycin on *kerA* expression. **Figure 6A** represents results obtained from bacterial strain FDB-3 in Luria-Bertani (LB) medium and feather medium (FM). **Figure 6B** represents results obtained from bacterial strain FDB-29 in Luria-Bertani (LB) medium and feather medium (FM). **Figure 6C** represents results obtained from bacterial strain FDB-36 in Luria-Bertani (LB) medium and feather medium (FM).

Detailed Description of the Invention

Amino acid sequences disclosed herein are presented in the amino to carboxy direction, from let to right. The amino and carboxy groups are not presented in the sequence. Nucleotide sequences are presented herein by single coding strand only, in the 5′ to 3′ direction, from left to right. Nucleotides

and amino acids are represented herein in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission, or (for amino acids) by three letter code, in accordance with 37 CFR §1.822 and established usage. *See, e.g.*, PatentIn User Manual, 99-102 (Nov. 1990) (U.S. Patent and Trademark Office, Office of the Assistance Commissioner for Patents, Washington, D.C. 20231); U.S. Patent No. 4,871,670 to Hudson *et al.*, at Col. 3, lines 2-=43 (applicants specifically intend that the disclosure of this and all other patent references cited herein be incorporated herein by reference).

A. DNA Encoding Keratinase Enzyme

DNA molecules which encode a keratinase enzyme are those which encode a protein capable of degrading a keratin source such as feathers. This definition is intended to encompass natural allelic variations in the DNA molecules. As used herein, "natural" or "native" DNA refers to sequences isolated from natural sources, as opposed to sequences created by chemical synthesis and not found in nature.

Hybridization conditions which will permit other DNA sequences which code on expression for a keratinase to hybridize to a DNA sequence as given herein are, in general, high stringency conditions. For example, hybridization of such sequences may be carried out under conditions represented by a wash stringency of 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS at 60°C or even 70°C to DNA disclosed herein in a standard *in situ* hybridization assay. *See*, J. Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (2nd. Ed. 1989) (Cold Spring Harbor Laboratory)). In general, DNA sequences which code for a keratinase and hybridize to the DNA sequence encoding the *Bacillus licheniformis* PWD-1 keratinase disclosed herein will be at least 65%, 70%, 75%, 80%, 85%, 90%, or even 95%

homologous or more with the sequence of the keratinase DNA disclosed herein.

Further, DNA sequences (or oligonucleotides) which code for the same keratinase as coded for by the foregoing sequences, but which differ in codon sequence from these due to the degeneracy of the genetic code, are also an aspect of this invention. The degeneracy of the genetic code, which allows difference nucleic acid sequences to code for the same protein or peptide, is well known in the literature. *See, e.g.*, U.S. Patent No. 4,757,006 to Toole *et al*. at Col. 2, Table 1.

DNA sequences (or oligonucleotides) which code for the same keratinase as coded for by the foregoing sequences, but which differ in codon sequence from these due to site directed mutagenesis are also contemplated by this invention. Site directed mutagenesis techniques useful for improving the properties of the keratinase enzyme are well known, as described below. *See, e.g.*, U.S. Patent No. 4,9873,192 to Kunkel.

As used herein, "*kerA*" refers to the 1.457 kilobase keratinase gene encoding keratinase and including the *kerA* pre/pro processing and secretion region. The nucleotide sequence for *kerA* gene is set forth in SEQ ID NO.:1. The amino acid sequence encoded by *kerA* is set forth in SEQ ID NO.:2. Also as used herein, "*kerA* pre/pro processing and secretion region" refers to the nucleotide sequence from nucleotide 215 to nucleotide 529 of the *kerA* gene, which comprises the pre-region (nucleotides 215-301) and the pro-region (nucleotides 302-529). The processing and secretion region of keratinase permit the cleavage and the extra-cellular secretion of the expressed protein. The pre-region of *kerA* encodes a signal peptide for secretion of the protein. The pro-region of *kerA* encodes a signal peptide which controls correct folding of the

peptide. The mature protein of *kerA* extends from nucleotide 530 to nucleotide 1351, and encodes the 274 amino acid keratinase.

B. Genetic Engineering Techniques

The production of cloned genes, recombinant DNA, vectors, transformed host cells, proteins and protein fragments by genetic engineering is well known. *See, e.g.*, U.S. Patent No.4,761,371 to Bell *et al.* at Col. 6 line 3 to Col. 9, line 65; U.S. Patent No. 4,877,729 to Clark *et al.* at Col. 4, line 38 to Col. 7, line 6; U.S. Patent No. 4,912,038 to Schilling at Col. 3, line 26 to Col. 14, line 12; and U.S. Patent No. 4,879,224 to Wallner at Col. 6, line 8 to Col. 8, line 59.

The DNA encoding keratinase may be made according to any of the know techniques. For example, the DNA may be constructed using the MUTA-GENE™ phagemid *in vitro* mutagenesis kit by BIO-RAD. The kit is based on the method described by Kunkel in U.S Patent No. 4,873,192. (*See also* T. Kunkel, *Proc. Natl Acad. Sci. USA* **82**:488 (1985); T. Kunkel *et al.*, *Methods in Enzymol.* **154**:367 (1987)). U.S. Patent No. 4,873,192 provides a very strong selected against the non-mutagenized strand of a double-stranded DNA. When DNA is synthesized in a *dut-ung*-double mutant bacterium, the nascent DNA carries a number of uracils in thymine positions as a result of the *dut* mutation, which inactivates the enzyme dUTPase and results in high intracellular levels of dUTP. The *ung* mutation inactivates uracil N-glycosylase, which allows the incorporated uracil to remain in the DNA. This uracil-containing strand is then used as the template for the *in vitro* synthesis of a complementary strand primed by an oligonucleotide containing the desired mutation. When the resulting double-stranded DNA is transformed into a cell with a proficient uracil N-glycosylase, the

uracil-containing strand is inactivated with high efficiency, leaving the non-uracil-containing survivor to replicate (*See generally,* BIO-RAD catalog number 170-3576 instruction manual).

The keratinase gene encompassing the DNA encoding keratinase as well as regulatory elements may be constructed by amplification of a selected, or target, nucleic acid sequence. Amplification may be carried out by any suitable means. *See generally,* D. Kwoh and T. Kwoh, *Am. Biotechnol. Lab.* **8**:14 (1990). Examples of suitable amplification techniques include, but are not limited to polymerase chain reaction, ligase chain reaction, strand displacement amplification (*see generally,* G. Walker *et al.*, *Proc. Natl. Acad. Sci. USA* **89**:392 (1992); G. Walker *et al.*, *Nucleic Acids Res.* **20**:1691 (1992)), transcription-based amplification (*see,* D. Kwoh *et al.*, *Proc. Natl. Acad Sci. USA* **86**:1173 (1989)), self-sustained sequence replication (or "3SR") (*see,* J. Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA* **87**:1874 (1990)), the Qβ replicase system (*see,* P. Lizardi *et al.*, *Biotechnology* **6**:1197 (1988)), nucleic acid sequence-based amplification (or "NASBA") (*see,* R. Lewis, *Genetic Engineering News* 12 **9**:1 (1992)), the repair chain reaction (or "RCR") (*see,* R. Lewis, *supra*), and boomerang DNA amplification (or "BDA") (see R. Lewis, *supra*). Polymerase chain reaction is currently preferred.

DNA amplification techniques such as the foregoing can involve the use of a probe, a pair of probes, or two pairs of probes which specifically bind to DNA encoding the desired target protein.

Polymerase chain reaction (PCR) may be carried out in accordance with known techniques. *See, e.g.,* U.S. Patents Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188. In general, PCR involves, first, treating a nucleic acid sample (e.g., in the presence of a heat stable DNA polymerase) with one

oligonucleotide primer for each strand of the specific sequence to be detected under hybridizing conditions so that an extension product of each primer is synthesized which is complementary to each nucleic acid strand, with the primers sufficiently complementary to each strand of the specific sequence to hybridize therewith so that the extension product synthesized from each primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and then treating the sample under denaturing conditions to separate the primer extension products from their templates if the sequence or sequences to be detected are present. These steps are cyclically repeated until the desired degree of amplification is obtained. Detection of the amplified sequence may be carried out by adding to the reaction product an oligonucleotide probe capable of hybridizing to the reaction product (e.g., an oligonucleotide probe of the present invention), the probe carrying a detectable label, and then detecting the label in accordance with known techniques, or by direct visualization on a gel.

Ligase chain reaction (LCR) is also carried out in accordance with known techniques. *See, e.g.*, R. Weiss, *Science* **254**:1292 (1991). In general, the reaction is carried out with two pairs of oligonucleotide probes: one pair binds to one strand of the sequence to be detected; the other pair binds to the other strand of the sequence to be detected. Each pair together completely overlaps the strand to which it corresponds. The reaction is carried out by, first, denaturing (e.g., separating) the strands of the sequence to be detected, then reacting the strands with the two pairs of oligonucleotide probes in the presence of a heat stable ligase so that each pair of oligonucleotide probes is ligated together, then separating the reaction product, and then cyclically

repeating the process until the sequence has been amplified to the desired degree. Detection may then be carried out in like manner as described above with respect to PCR.

A vector is a replicable DNA construct. Vectors are used herein either to amplify DNA encoding a proteinase or keratinase as given herein and/or to express DNA which encodes a proteinase or keratinase as given herein. An expression vector is a replicable DNA construct in which a DNA sequence encoding a proteinase or keratinase is operably linked to suitable control sequences capable of effecting the expression of the proteinase or keratinase in a suitable host. The need for such control sequences will vary depending upon the host selected. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation.

Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants.

Vectors comprise plasmids, viruses (e.g., adenovirus, cytomegalovirus), phage, and integratable DNA fragments (i.e., fragments integratable into the host genome by recombination). The vector replicates and functions independently of the host genome, or may, in some instances, integrate into the genome itself. Expression vectors should contain a promoter and RNA polymerase binding sites which are operably linked to the gene to be expressed and are operable in the host organism.

DNA regions are operably linked or operably associated when they are functionally related to each

other. For example, a promoter is operably linked to or operably associated with a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

Transformed host cells are cells which have been transformed or transfected with vectors containing a DNA sequence as disclosed herein constructed using recombinant DNA techniques. Transformed host cells ordinarily express the proteinase or keratinase, but host cells transformed for purposes of cloning or amplifying the proteinase or keratinase DNA do not need to express the proteinase or keratinase. Suitable host cells can include host cells known to those skilled in the art, such as for example prokaryote host cells including *Bacillus subtilis*.

In the methods and systems of the present invention, *Bacillus subtilis* host cells are preferred. *Bacillus subtilis* is capable of secreting enzymes extracellularly. (*See generally*, Priest, *Bacterial. Rev.* **41**:711 (1977) and Doi *et al.*, *Trends Biotechnol*. Sept. 232 (1986). This feature allows this bacterium to serve as a host cell for expression and secretion of foreign proteins in the medium, which can be conveniently rendered to downstream processing and utilization. The *Bacillus subtilis* system has not be widely utilized, because either the inserted gene is poorly regulated in general, or foreign proteins are likely to be hydrolyzed by high levels of proteases produced by *Bacillus subtilis*. *Bacillus subtilis* has six extracellular proteases, neutral protease A, subtilisin (or "alkaline protease"), extracellular protease, metalloprotease, bacillopeptidase F, and neutral protease B. To overcome these problems, protease-deficient strains of *Bacillus subtilis* have been developed. (*See generally*, Doi *et al.*, *Trends*

Biotechnol. **4**:232 (1986) and Wu *et al. J. Bacteriol.* **173**:4952 (1991)). *Bacillus subtilis* deficient in only neutral protease, DB101, has been developed. A *Bacillus subtilis* strain deficient in two extracellular protease, namely neutral protease and alkaline protease, and known as DB104 has been developed. A *Bacillus subtilis* strain deficient in five proteases, known as GP263, has been developed and has eliminated much of the total extracellular protease activity. A *Bacillus subtilis* strain deficient in all six extra-cellular proteases, WB600, has also been constructed. Currently, DB104, or *Bacillus subtilis* deficient in two extracellular proteases, is the preferred strain for the host cells employed in the present invention.

Vectors for use in *Bacillus subtilis* host cells have been constructed. (*See generally*, Steinmetz *et al.*, *Mol. Gen. Genet.* **200**:220 (1985), Crutz *et al.*, *J. Bacteriol.* **172**:1043 (1990), and Wu *et al.*, (1991) *supra.*) Preferably, *Bacillus subtilis* is transformed using vectors generated from pUB18 or pUB18-P43 plasmids. A promoter commonly used in these recombinant expression vectors include the strong vegetative promoter P43. The promoter is operably associated to the DNA encoding the keratinase, i.e., they are positioned so as to promote transcription of keratinase messenger RNA from the DNA.

The hyperexpression of keratinase has been observed using the *Bacillus subtilis* system where the *kerA* pre/pro processing and secretion region is inserted upstream of the DNA encoding keratinase. Hence, this is the preferred embodiment of the instant invention.

C. Production of Keratinase Enzyme

As noted above, keratinase enzyme can be made by culturing a host cell as described above under conditions that permit expression of the encoded

keratinase, and collecting the expressed keratinase. The host cell may be cultured under conditions in which the cell grows, and then cultured under conditions which cause the expression of the encoded keratinase, or the cells may be caused to grow and express the encoded keratinase at the same time. The keratinase may be fused to an appropriate secretory leader sequence and secreated into the culture media and collected from the media, or the keratinase may be expressed intracellularly, the cells then lysed, and the keratinase collected from the cell lysate. Preferably, the enzyme is produced into the culture medium and collected therefrom. In general, any suitable techniques for culturing and expressing a transgenic protein may be used, as will be appreciated by those skilled in the art.

For example, the transformed *Bacillus subtilis* host cells may be cultured in Luria-Bertoni or feather medium, into which the expressed keratinase enzyme is secreted and from which the keratinase may be collected. The *Bacillus subtilis* host cells are typically cultured at temperatures ranging from 30 to 45°C. The expressed enzyme may be collected from the medium according to techniques widely known in the art. For example, the enzyme can be concentrated by ultrafiltration or ammonium sulfate precipitation, and purified by various chromatographic methods, as described in Lin *et al.*, *Applied Environmental Microbiology* **58**:3271 (1992).

According to one preferred embodiment of the present invention, keratinase is produced by (*a*) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith; and (*b*) collecting keratinase enzyme from said cell culture. According to one preferred embodiment, the vector DNA further

comprises DNA encoding a *kerA* processing and secretion region. More preferably, the vector DNA further comprises a promoter, such as a P43 promoter, located upstream of the DNA encoding a *kerA* processing and secretion region. According to one preferred embodiment, the promoter is positioned in the same orientation as the DNA encoding the *Bacillus licheniformis* PWD-1 keratinase enzyme.

D. Recombinant DNA and System for Expression of a Heterologous DNA

The present invention also provides a recombinant DNA and host cell for expressing a heterologous DNA encoding an enzyme or protein. Typically the heterologous DNA encoding an enzyme comprises a heterologous DNA encoding a proteinase. Preferably, the heterologous DNA encoding an enzyme comprises a heterologous DNA encoding a keratinase. Examples of suitable heterologous DNA encoding enzymes for use in the present invention include but are not limited to proteases, amylase, lipase, hexose isomerase, β-gluconase, and phytase.

According to the present invention, the recombinant DNA comprises vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and the heterologous DNA encoding an enzyme or protein. The vector DNA typically comprises a promoter. Any suitable promoter capable of regulating the expression of the heterologous DNA in the selected host cell may be employed. Preferably, the promoter is a P43 promoter. In the preferred embodiment of the recombinant DNA of the present invention, the promoter is located upstream of the DNA encoding the *kerA* pre/pro processing and secretion region and is in the same orientation as the heterologous DNA encoding the enzyme or protein.

The recombinant DNA may be transfected into a host cell to provide a host cell capable of expressing the heterologous DNA. Suitable host cells include those host cells discussed hereinabove in connection with the expression and secretion of keratinase. The preferred host cell is *Bacillus subtilis*, and particularly the *Bacillus subtilis* strain which is deficient in both neutral and alkaline cellular proteases. The recombinant DNA of the present invention and the host cell provide a *Bacillus* system for the expression and secretion of an enzyme or protein encoded by a heterologous DNA.

E. Methods of Expressing Heterologous DNA

The present invention also provides methods of expressing a heterologous DNA encoding an enzyme or protein. The methods of the present invention include (*a*) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme or protein, and (*b*) collecting enzyme or protein from the *Bacillus subtilis* host cell culture or cell culture medium. The recombinant DNA and host cell of the present invention are described in further detail hereinabove.

The following examples are provided to illustrate the present invention, and should not be construed as limiting thereof. In these examples, "g" means grams, "μg" means micrograms, "l" means liters, "ml" means milliliters, "g/l" means grams per liter, "μg/ml" means micrograms per milliliter, "°C" means degrees Centigrade, "Km" means kanamycin.

Bacillus licheniformis PWD-1 has the accession number ATCC 53757. *Bacillus licheniformis* PWD-1 was grown on either 1) feather medium consisting of 0.5 g/l of sodium chloride, 0.1 g/l magnesium

chloride hexahydrate, 0.06 g/l calcium chloride, 0.7 g/l KH_2PO_4, 1.4 g/l K_2HPO_4, 1.0 g/l tryptone, and 10 g/l chopped feathers at pH 7.0; or 2) Luria-Bertani ("LB") medium at 50°C. *Bacillus subtilis* DB104 is grown according to Kawamura and Doi, *J. Bacteriol.* **160**:442 (1984) and is deficient in both alkaline and neutral extracellular proteases. Specifically, *B. subtilis* DB104 was grown at 37°C on LB medium. *B. subtilis* DB104 carrying plasmid pUB18 or its derivatives, Km was added to the medium at a final concentration of 20 μg/ml. *Escherichia coli* INVαF' and PCR cloning vector, pCRII, were purchased from Invitrogen Corporation, San Diego, California. *E. coli* INVαF' was grown at 37°C on LB medium supplemented with 50 μg/ml ampicillin. TBAB plates containing 20μg Km/ml were obtained from Difco Laboratories, Detroit, Michigan and used for routine transformation. A skim milk-feather powder plate (containing 5% skim milk, 0.5% feather powder, 1% agar, and 20μg Km/ml) were used to screen colonies producing keratinase. Transformed *B. subtilis* strains were grown at 37°C on LB medium or feather medium.

EXAMPLE 1

DNA Manipulations

Mini-preparation of plasmids of pUB18, pUB18-P43 and their derivatives are prepared by rapid alkaline sodium dodecyl sulfate method, according to the method of Rodriguez, <u>Recombinant DNA Techniques</u>, Addison-Wesley Publishing Co., (1983), the disclosure of which is incorporated herein by reference in its entirety. The 1.4 kb *kerA* fragment is cloned into polylinker site of plasmid pCRII and stored in *E. Coli* INVαF' as described previously by Lin *et al.*, *Applied Environmental Microbiology* 61:1469 (1995), the disclosure of which is incorporated herein by reference in its entirety. After *E. coli* INVαF' cells are grown on LB medium overnight, plasmid pCRII with *kerA* is

extracted by several mini-preparations, pooled and excised for *kerA* by *XbaI* and *SpeI* digestion. The digestion mixture is applied on 1.2% agarose gel electrophoresis for separation. *kerA* band is cut out, and extracted from the gel by using an Elu-Quik DNA purification kit purchased from Schleicher & Schuell, Keene, New Hampshire. The extraction is carried out following the manufacturer's instruction. All restriction enzymes are the products of Promega Corporation, Madison, Wisconsin. The construction of plasmid pLB3 contianing *kerA* is set forth in **Figure 1**. *Km*^{r} represents the kanamycin resistance gene. Arrows indicate the orientations of the genes.

EXAMPLE 2

Construction of Vectors

Plasmid pUB18-P43 is created by inserting a DNA fragment (~300 bp) containing vegetative promoter P43 as described in Wang, *et al.*, *Journal of Biological Chemistry* **259**:8619 (1984), adjacent to the polycloning site of pUB18. Both plasmids pUB18 and pUB18-P43 have the same polycloning site available for gene insertion. When the plasmids are digested by *HindIII* (5'-AAGCTT-3'), four-base overhangs (5'-AGCT-3') are generated on both ends. Partial fill in with nucleotides A and G generated two-nucleotides overhangs (5'-AG-3') at the ends of the linearized vectors. The 1.4-kb *kerA* fragment in pCRII flanking by *XbaI* (5'-TCTAGA-3') and *SpeI* (5'-ACTAGT-3') recognization site was excised by *XbaI-SpeI* digestion. The same single-strand overhangs (5'-CTAG-3') are generated at both ends. Again, partial fill in with nucleotides T and C created another two-nucleotide overhangs (5'-CT-3') at both ends of the insert. These two separate treatments produced complementary overhangs on the vectors and insert as illustrated in **Figure 1**. Vector and insert in a molar ratio of 1:2 are mixed and ligated according

to the method of Sambrook, *et al.*, Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, the disclosure of which is incorporated herein by reference.

The structures of plasmids pLB3, pLB29, and pLB36 are set forth in **Figure 2**.

EXAMPLE 3

Cloning and Screening

The linearized pUB18 and pUB18-P43 created by *Hind*III digestion were flanked by overhangs 5'-AGCT-3', which is not complementary with the overhangs on *kerA* fragment generated by *XbaI-SpeI* digestion. However, the fill-in treatments on vectors by AG and on insert by CT generated complementary two-nucleotide overhangs between vectors and *kerA* fragment to facilitate the ligation. Fill in also prevented linearized vector from religation, which reduced background colonies dramatically during the transformants screening. Using skim milk-feather powder plates proved to be an efficient means of selecting transformants capable of expressing *kerA*.

EXAMPLE 3

Preparation of *B. subtilis* Competent Cells

B. subtilis DB104 competent cells are prepared as described in Dubnau *et al.*, *Journal of Molecular Biology* **56**:209 (1971), the disclosure of which is incorporated herein by reference in its entirety. *B. Subtilis* cells grown overnight on TBAB plates are inoculated with 2 ml of SP1 medium according to J Spizizen, *Proc. Natl. Acad. Sci. USA* **44**:1072 (1958) and Dubnau *et al.*, *Journal of Molecular Biology* **56**:209 (1971). The SP1 medium is prepared with 0.2% $(NH_4)_2SO_4$, 1.4% K_2HPO_2, 0.6% KH_2PO_4, 0.1% sodium citrate·$2H_2O$, 0.02% $MgSO_4$, 0.02% casamino acids, 0.1%

yeast extract, 0.005% tryptophan. One ml of pre-filtrated (0.2μ membrane) 50% glucose solution per 100 ml of SP1 medium is added after the medium is autoclaved. Cells are grown at 37°C for 3.5 to 4 hours with rapid shaking at 300 rpm. A 0.5 ml culture of SP1 medium is then transferred to 4.5 ml SP2 medium (SP1 medium with additional 0.5 mM $CaCl_2$ and 2.5 mM $MgCl_2$), and grown for an additional 90 minutes. Thereafter, 50 μl of EGTA solution (100 mM EGTA, pH 7.0) is added to the SP2 medium. The cells are ready for transformation after shaking for 10 minutes.

EXAMPLE 4

Transformation of *B. subtilis* DB104 and Screening for Colonies Harboring Plasmid

Ligated DNA in 50 μl is added to 0.5 ml of freshly prepared *B. subtilis* DB104 competent cells. After shaking at 200 rpm at 37°C for 90 minutes, cells are plated on TBAB plates with 20 μg Km/ml, and incubated at 37°C overnight. Colonies grown on TBAB plates are transferred to skim-milk-agar plates for further selection. The colonies having clear haloes are selected for plasmid isolation and analysis.

Transformation of *B. subtilis* using ligated pUB18-*kerA* and pUB18-P43-*kerA* DNA yielded hundreds of colonies on TBAB plates. Thirty six from each group are randomly selected and transferred onto skim milk-agar feather powder plates for a secondary selection. Seven colonies from pUB18-*kerA* transformant group and six colonies from pUB18-P43-*kerA* transformant group produced clear halos around colonies in 10 hour incubation at 37°C, while DB104/pUB18 and DB104/pUB18-P43 cells as controls did not show any sign of protein hydrolysis even after 48 hours. Those transformants cells are then grown in LB medium containing 20 μg km/ml for 3 hours. Cells in 2 ml culture from each clone are used for plasmid isolation.

EXAMPLE 5

Analysis of Plasmid Constructs

All plasmids isolated from halo-forming colonies displayed a 1.4 kb increase in size. When the plasmids were used as templates for PCR amplifications, 1.4 kb fragments were produced in the reactions priming by Primer I and Primer II. These results confirmed that the increase in size by 1.4 kb is due to the insertion of *kerA*.

Plasmids pLB3, pLB29, and pLB 36 represent all new vectors isolated from halo-forming colonies. In fact, pLB3 represents all plasmids isolated from pUB18-*kerA* group because all of them have the *kerA* in the same orientation. In the pUB-P43-*kerA* group, pLB29 and pLB36 represent two opposite orientations of *kerA*.

To determine the orientation of *kerA* in the plasmids, Primer III was combined with either Primer I or Primer II to perform PCR amplifications. When pLB3 and pLB36 as templates, and Primer I and Primer III are used, PCR amplified a 1.5 kb fragment and a 1.8 kb fragment respectively. The increases in size were due to the amplification of an additional 52 bp from original pUB18 and ~350 bp from original pUB18-P43. The presence of PCR products also proved that *kerA* in pLB3 and pLB36 have the same orientation, and that they have the same orientation as the kanamycin resistance gene (Km^r) on the vectors. PCR using pLB29 template and Primer I and Primer III did not produce any major DNA fragment. However, when Primer I is replaced by Primer II, a 1.8 kb fragment is observed on the agarose gel. These results indicate that *kerA* in pLB29 is in the same orientation with P43 promoter, but opposite to Km^r.

EXAMPLE 6

Identification of *kerA* in Plasmids

The newly constructed plasmids are digested by *XbaI*, followed by 1.2% agarose gel analysis.

Plasmids with a 1.4 kb size increase are applied to PCR amplifications. Three PCR primers are synthesized: Primer I (5'-CTCCTGCCAAGCTGAAGC-3', 18 mers) (SEQ ID NO.:3) and Primer II (5'-GATCATGGAACGGATTC-3', 17 mers) (SEQ ID NO.:4), which are homologous to the upstream and downstream of *kerA*, respectively and Primer III (5'-GCCGTCTGTACGTTCCTAAG-3', 20 mers) (SEQ ID NO.:5) which is derived from the upstream DNA sequence of the polycloning site on pUB18 and pUB18-P43. PCR amplifications with any two of the given primers are performed as described in Lin *et al.*, *Applied Environmental Microbiology* 61:1469 (1995)., the disclosure of which is incorporated herein by reference in its entirety; except that the newly constructed plasmids are used as templates. Approximately 156 ng plasmid DNA is used as the template in each PCR reaction.

EXAMPLE 7

Expression of *kerA* in LB and Feather Media

Five strains, *B. subtilis* DB104/pUB18, FDB-3 (DB104/pLB3), FDB-29 (DB104/pLB29), FDB-36 (DB104/pLB36), and *B. licheniformis* PWD-1 grew rapidly in LB medium. At 36 hours, 40 μl of supernatant from each medium is loaded into small wells on milk-agar plate, and incubated at 50°C overnight. Hydrolysis haloes are only observed around the wells in which supernatants from FDB-3, FDB-29, and FDB-36 are loaded. This result is confirmed by the azo-keratin hydrolysis assay, when a 0.2 ml sample of each medium is taken at every 4 hours and determined for its keratinolytic activity. Again media from all three strains showed strong activities against azokeratin, and FDB-29 gives the highest activity among all the three transformants. Both PWD-1 and DB104/pUB18 media showed no proteolytic activities.

All five strains are also tested in feather media. PWD-1 and FDB-29 grew rapidly and reach their highest keratinase activity in approximately 72 hours. FDB-3, and FDB-36 did not display significant keratinolytic activities until the third day, reaching their highest activities at least 24 hours later than FDB-29 did. FDB-29 still demonstrated the highest activity, which was 3 to 4 fold higher than that of PWD-1 grown on feather media at 50°C. PWD-1 showed positive results only in feather media. DB104, the host strain, does not produce keratinase in either LB or feather media.

In feather media all these new strains, FDB-3, FDB-29, and FDB-36 yielded more keratinolytic activity when kanamycin was not added in the medium. FDB-29 produced more keratinase in LB medium without this antibiotic. FDB-3 and FDB-36 however, demonstrated higher keratinolytic activity in LB medium when kanamycin was added.

EXAMPLE 8

Detection of Keratinase Activity

Two methods, hydrolysis of azokeratin and milk-agar plate assay, are used to detect keratinase activity according to the methods described in Lin *et al.*, *Applied Environmental Microbiology* **58**:3271 (1992), the disclosure of which is incorporated herein by reference in its entirety. SDS gel electrophoresis is conducted as described in Laemmili, *et al.*, *Nature* **227**:680 (1970), the disclosure of which is incorporated herein by reference in its entirety. Purified keratinase is used to generate anti-keratinase serum in rabbits by the standard method described in Harlow *et al.*, Antibodies, A Laboratory Manual (1988) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. This anti-serum which precipitates with keratinase is used to detect the enzyme in agar gel. DNA restriction

and agarose gel electrophoresis are performed as described by Sambrook *et al.*, Molecular Cloning, A Laboratory Manual 2nd ed. (1988).

EXAMPLE 9

Confirmation of Expression of *kerA*

Active keratinase was produced by FDB-3, FDB-29, and FDB-36 in LB and feather media. This has been confirmed by milk-agar plate (contianing 4% evaporated skim-milk, 1.5% agar, and 0.02% sodium azide) assay. **Figure 3** illustrates the detection of proteolytic activty by formation of hydrolysis haloes on milk-agar plates. Plate A contains cell-free culture supernatants from feather medium. *Bacillus licheniformis* PWD-1 and FDB-29 samples were taken at 72 hours, FDB-3, FDB-36, and DB104/pUB18 samples were taken at 96 hours. Plate B contains 36-hour cell-free culture supernatants from LB medium. PWD-1 was grown at 50°C and all others were grown at 37°C.

Confirmation of the production of active keratinase by FDB-3, FDB-29 and FDB-36 in LB and feather media was also obtained by azokeratine hydroylsis as illustrated by **Figure 4**. The assay was carried out in 500 ml flask with 150 ml medium. Seed cultures of FDB-3, FDB-29, FDB-36 and DB104/pUB18 were grown in 10 ml LB medium with 20 μg Km/ml for 4 hours, and 1 ml of each was inocultated to 150-ml flask feather and LB media. Seed culture of PWD-1 (10 ml) were grown on LB and feather media for overnight firstly, and 1 ml of each was inoculated to LB and feather media, respectively. No kanamycin was added into feather medium or PWD-1 growth media. Keratinolytic activity was measured according to the methods described in Lin *et al.*, *Appl. Environ. Microbiol.* 58:3271 (1992).

Confirmation of the production of active keratinase by FDB-3, FDB-29 and FDB-36 in LB and

feather media was also obtained by immuno-precipitation assay as illustrated by **Figure** 5. The rabbit anti-keratinase serum was loaded into the holes in the center of each plate. Plate A contains cell-free culture supernatnats from feather medium. *Bacillus licheniformis* PWD-1 and FDB-29 sampels were taken at 72 hours, FDB-3, FDB-36, and DB104/pUB18 samples were taken at 96 hours. Plate B contains 36-hour cell-free culture supernatants from LB medium.

The double immuno-diffusion results indicate that FDB-3 , FDB-29, and FDB-36 produced keratinase in both LB and feather media. PWD-1 in LB media and DB104 in both feather and LB media have negative responses in this immuno-precipitation assay.

Confirmation of keratinase activity was also achieved using SDS-polyacrylamide gel electrophoresis. The 33 kDa keratinase bands appeared on SDS-polyacrylamide gel when the media of FDB-3, FDB-29, and FDB-36 are used.

EXAMPLE 10

Effects of Promoter Orientation

As discussed in **Example 6**, PCR amplification analysis illustrated that P43 was installed upstream of *kerA* in pLB29 and in the same orientation as as *kerA*. pLB36 has the P43 promoter in the opposite orientation from *kerA* and pLB3 does not contain the P43 promoter. The results of the keratinolytic activity of FDB-29, FDB-36, and FDB-3 cells demonstrate that the P43 promoter greatly enhanced the expression of *kerA*. Rapid cell growth of FDB-29 cells, associated with keratinolytic activity increase, was observed in feather medium. In contrast, FDB-3 and FDB-36 in feather medium show a long adaptive period, and produce most of their enzymes after 4 days of culture. Although the inventors do not wish to be bound by any particular theory, it appears that FDB-3 and FDB-36

underwent an induction process, which resulted in the eventual expression of *kerA*.

EXAMPLE 11

Effects of Orientation of Kanamycin Resistance Gene

The kanamycin resistance gene (*Km*^{r}) carried by plasmid expresses in response to kanamycin in the medium, and has an influence on the expression of *kerA*. In the presence of kanamycin, in both LB and feather media, FDB-29 produced slightly low activities, as reported in **Figure 6B**. The decrease in *kerA* expression may be due to the generation of antisence RNA resulting from the readthrough of the kanamycin resistance gene. For FDB-3 and FDB-36, the increase in expression of *kerA* may also be caused by the same readthrough of the kanamycin resistance gene, since *kerA* and the kanamycin resistance gene in these two vectors are in the same orientation. The results for FDB-3 and FDB-36 are reported in **Figures 6A** and **6C** respectively. The same increases were not found when FDB-3 and FDB-36 were grown in the feather medium. It is possible that the induction of *kerA* expression is crucial when they are grown on feathers. No keratinolytic activity was produced by *Bacillus licheniformis* PWD-1 in LB medium.

EXAMPLE 12

Secretion of Keratinase in Protease-Deficient *Bacillus subtilis*

The DB104 host cells employed in the foregoing experiments are deficient in two major extracellular proteases, neutral and alkaline proteases. The results of keratinolytic activity indicate that DB104 is able to express *kerA* originating from *Bacillus licheniformis* strain and secrete active keratinase into the medium at a high level. Because *kerA* pre/pro processing and secretion region exist upstream of the keratinase structure gene, premature

keratinase in the cell must have been processed to active enzyme. These results demonstrate that the *kerA* pre/pro processing and secretion region is recognized and processed in DB104 even though it is deficient in two major cellular proteases.

A similar *Bacillus subtilis*, WB600, which is deficient in six cellular proteases was also tested for expression of *kerA* in pLB29. Low enzyme activity was produced in LB medium. These results suggest that for effective production of foreign protein, the host cell with high levels of extracellular proteases is harmful, but a low level of proteolytic process may be necessary for activating enzymes by limited proteolysis.

The foregoing is illustrative of the present invention and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Shih, Jason C.H.
Lin, Xiang
Wong, Sui-Lam

(ii) TITLE OF INVENTION: Method For Expressing and Secreting Keratinase

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Kenneth D. SIbley
(B) STREET: P.O. Drawer 31107
(C) CITY: Raleigh
(D) STATE: North Carolina
(E) COUNTRY: USA
(F) ZIP: 27622

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Sibley, Kenneth D.
(B) REGISTRATION NUMBER: 31,665
(C) REFERENCE/DOCKET NUMBER: 5051-304

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (919) 420-2200
(B) TELEFAX: (919) 881-3175

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1457 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 215..1351

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 530..1351

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 215..301
(D) OTHER INFORMATION: /note= "pre-region of keratinase"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 302..529
(D) OTHER INFORMATION: /note= "pro-region of keratinase"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ACT TAT TTG GGA AGC TCC TTC TAC TAT GGG AAA GGT CTG ATC AAT GTC      1336
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 379 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Val Ile Asn Met Ser Leu Gly Gly Ala Ser Gly Ser Thr Ala Met Lys
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            155                 160                 165

Ala Lys Tyr Asp Ser Val Ile Ala Val Gly Ala Val Asp Ser Asn Ser
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Asn Arg Ala Ser Phe Ser Ser Val Gly Ala Glu Leu Glu Val Met Ala
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Pro Gly Ala Gly Val Tyr Ser Thr Tyr Pro Thr Asn Thr Tyr Ala Thr
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Leu Asn Gly Thr Ser Met Val Ser Pro His Val Ala Gly Ala Ala Ala
                220                 225                 230

Leu Ile Leu Ser Lys His Pro Asn Leu Ser Ala Ser Gln Val Arg Asn
            235                 240                 245

Arg Leu Ser Ser Thr Ala Thr Tyr Leu Gly Ser Ser Phe Tyr Tyr Gly
        250                 255                 260

Lys Gly Leu Ile Asn Val Glu Ala Ala Ala Gln
    265                 270
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTCCTGCCAA GCTGAAGC 18

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATCATGGAA CGGATTC 17

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCGTCTGTA CGTTCCTAAG 20

That Which Is Claimed Is:

1. A *Bacillus subtilis* host cell containing a recombinant DNA molecule, wherein said recombinant DNA molecule comprises vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith, and capable of expressing and secreting keratinase.

2. The *Bacillus subtilis* host cell according to Claim 1, wherein said vector DNA of said recombinant DNA molecule further comprises a *kerA* pre/pro processing and secretion region.

3. The *Bacillus subtilis* host cell according to Claim 1, wherein said vector DNA of said recombinant DNA molecule further comprises a promoter.

4. The *Bacillus subtilis* host cell according to Claim 3, wherein said promoter is positioned upstream from said DNA encoding said *kerA* pre/pro processing and secretion region, and is operatively associated therewith.

5. The *Bacillus subtilis* host cell according to Claim 4, wherein said promoter is in the same orientation as said DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme.

6. The *Bacillus subtilis* host cell according to Claim 3, wherein said promoter is a P43 promoter.

7. The *Bacillus subtilis* host cell according to Claim 1, wherein said *Bacillus subtilis* host cell is deficient in at least one protease.

8. The *Bacillus subtilis* host cell according to Claim 1, wherein said *Bacillus subtilis* host cell is deficient in both neutral and alkaline proteases.

9. A method of producing keratinase enzyme comprising:

(*a*) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith; and

(*b*) collecting keratinase enzyme from said cell culture.

10. The method according to Claim 9, wherein said vector DNA of said recombinant DNA molecule further comprises a *kerA* pre/pro processing and secretion region.

11. The method according to Claim 9, wherein said vector DNA of said recombinant DNA molecule further comprises a promoter.

12. The method according to Claim 11, wherein said promoter is positioned upstream from a *kerA* pre/pro processing and secretion region and is operatively associated therewith.

13. The method according to Claim 11, wherein said promoter is in the same orientation as said DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme.

14. The method according to Claim 11, wherein said promoter is a P43 promoter.

15. The method according to Claim 9, wherein said step of collecting keratinase enzyme comprises separating said enzyme from said cell culture by a method selected from the group consisting of ultrafiltration and ammonium sulfate precipitation.

16. An expression and secretion system for keratinase enzyme comprising:

(*a*) a *Bacillus subtilis* host cell, and

(*b*) a recombinant DNA molecule comprising vector DNA, DNA encoding a *ker A* pre/pro processing and secretion region, and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith.

17. The expression and secretion system according to Claim 16, wherein said vector DNA of said recombinant DNA molecule further comprises a promoter.

18. The expression and secretion system according to Claim 17, wherein said promoter is located upstream from said *kerA* pre/pro processing and secretion region and operatively associated therewith.

19. The expression and secretion system according to Claim 17, wherein said promoter is in the same orientation as said DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme.

20. The expression and secretion system according to Claim 17, wherein said promoter is a P43 promoter.

21. The expression and secretion system according to Claim 16, wherein said *Bacillus subtilis* host cell is deficient in at least one protease.

22. The expression and secretion system according to Claim 16, wherein said *Bacillus subtilis* host cell is deficient in both neutral and alkaline proteases.

23. A recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme, wherein said heterologous DNA encoding said enzyme is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 keratinase enzyme.

24. The recombinant DNA molecule according to Claim 23, wherein said heterologous DNA encoding an enzyme comprises a heterologous DNA encoding a proteinase.

25. The recombinant DNA molecule according to Claim 23, wherein said heterologous DNA encoding an enzyme comprises a heterologous DNA encoding a keratinase.

26. The recombinant DNA molecule according to Claim 23, wherein said vector DNA further comprises a promoter.

27. The recombinant DNA molecule according to Claim 26, wherein said promoter is positioned upstream from said DNA encoding said *kerA* processing and secretion region, and is operatively associated therewith.

28. The recombinant DNA molecule according to Claim 26, wherein said promoter is in the same orientation as said heterologous DNA encoding said enzyme.

29. The recombinant DNA molecule according to Claim 26, wherein said promoter is a P43 promoter.

30. A *Bacillus subtilis* host cell containing a recombinant DNA molecule, wherein said recombinant DNA molecule comprises vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme; wherein said heterologous DNA encoding said enzyme is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 keratinase enzyme, and wherein said host cell is capable of expressing and secreting an enzyme encoded by said heterologous DNA.

31. The *Bacillus subtilis* host cell according to Claim 30, wherein said heterologous DNA encoding an enzyme comprises a heterologous DNA encoding a proteinase.

32. The *Bacillus subtilis* host cell according to Claim 30, wherein said heterologous DNA encoding an enzyme comprises a heterologous DNA encoding a keratinase.

33. The *Bacillus subtilis* host cell according to Claim 30, wherein said vector DNA of said recombinant DNA molecule further comprises a promoter.

34. The *Bacillus subtilis* host cell according to Claim 33, wherein said promoter is positioned upstream from said DNA encoding said *kerA* processing and secretion region, and is operatively associated therewith.

35. The *Bacillus subtilis* host cell according to Claim 33, wherein said promoter is in the same orientation as said heterologous DNA encoding said enzyme.

36. The *Bacillus subtilis* host cell according to Claim 33, wherein said promoter is a P43 promoter.

37. The *Bacillus subtilis* host cell according to Claim 30, wherein said *Bacillus subtilis* host cell is deficient in at least one protease.

38. The *Bacillus subtilis* host cell according to Claim 30, wherein said *Bacillus subtilis* host cell is deficient in both neutral and alkaline proteases.

39. A method of producing an enzyme comprising the steps of:

(*a*) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme, wherein said heterologous DNA encoding said enzyme is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 keratinase enzyme; and

(*b*) collecting enzyme from said *Bacillus subtilis* host cell culture.

40. The method according to Claim 39, wherein said heterologous DNA encoding said enzyme comprises a heterologous DNA encoding a proteinase.

41. The method according to Claim 39, wherein said heterologous DNA encoding said enzyme comprises a heterologous DNA encoding a keratinase.

42. The method according to Claim 39, wherein said vector DNA further comprises a promoter.

43. The method according to Claim 42, wherein said promoter is positioned upstream from said DNA encoding said *kerA* processing and secretion region, and is operatively associated therewith.

44. The method according to Claim 42, wherein said promoter is in the same orientation as said heterologous DNA encoding said enzyme.

45. The method according to Claim 42, wherein said promoter is a P43 promoter.

46. The method according to Claim 39, wherein said step of collecting said enzyme comprises separating said enzyme from said cell culture by a method selected from the group consisting of ultrafiltration and ammonium sulfate precipitation.

47. An expression and secretion system for an enzyme comprising:

(*a*) a *Bacillus subtilis* host cell, and

(*b*) a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme, wherein said heterologous DNA encoding said enzyme is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 keratinase enzyme.

48. The expression and secretion system according to Claim 47, wherein said heterologous DNA encoding said enzyme comprises a heterologous DNA encoding a proteinase.

49. The expression and secretion system according to Claim 47, wherein said heterologous DNA encoding said enzyme comprises a heterologous DNA encoding a keratinase.

50. The expression and secretion system according to Claim 47, wherein said vector DNA of said recombinant DNA molecule further comprises a promoter.

51. The expression and secretion system according to Claim 50, wherein said promoter is located upstream from said *kerA* pre/pro processing and secretion region, and is operatively associated therewith.

52. The expression and secretion system according to Claim 50, wherein said promoter is in the same orientation as said heterologous DNA encoding said enzyme.

53. The expression and secretion system according to Claim 50, wherein said promoter is a P43 promoter.

54. The expression and secretion system according to Claim 47, wherein said *Bacillus subtilis* host cell is deficient in at least one protease.

55. The expression and secretion system according to Claim 47, wherein said *Bacillus subtilis* host cell is deficient in both neutral and alkaline proteases.

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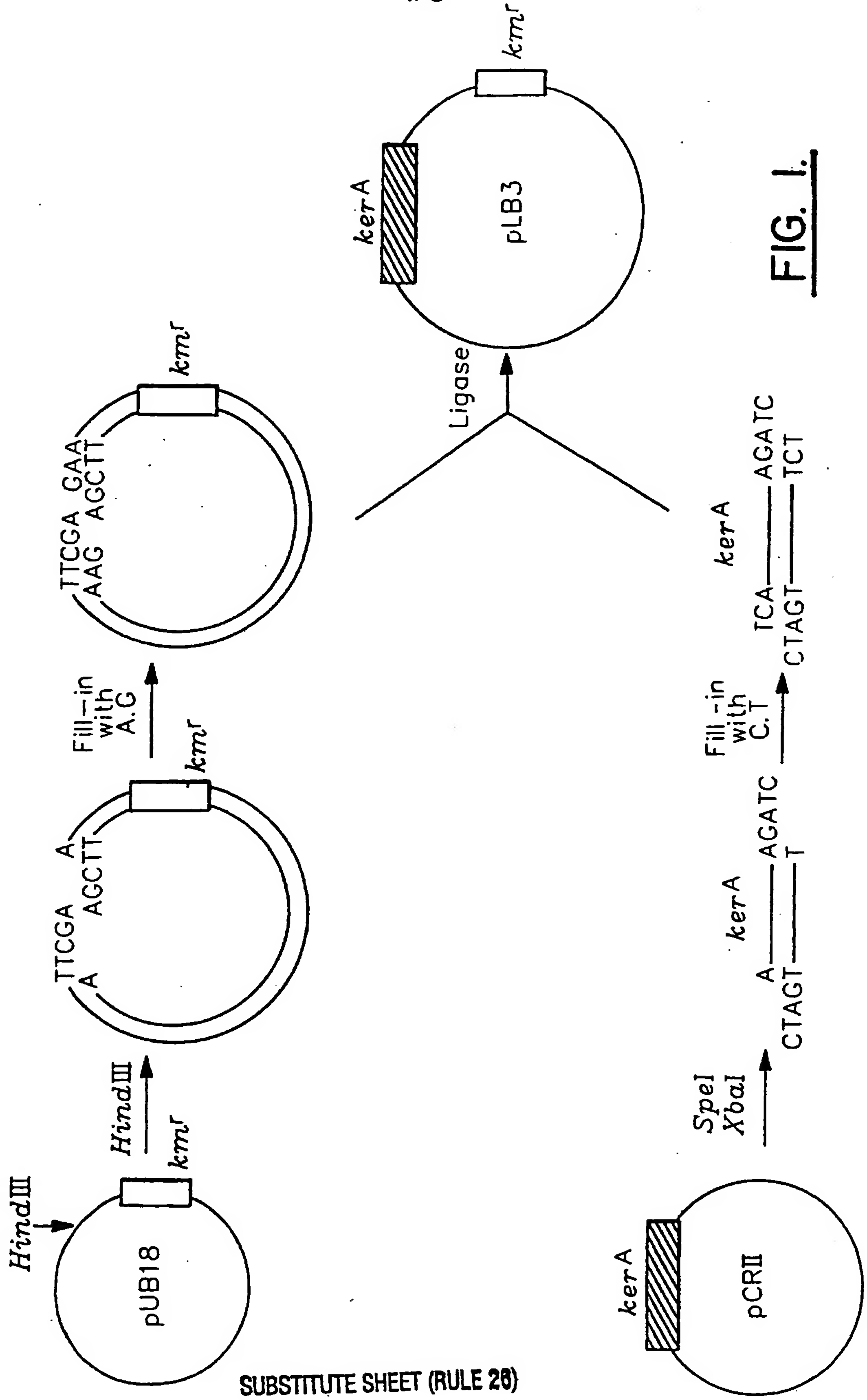
pUB18
HindIII
kmr
HindIII
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A
A
AGCTT
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AAG AGCTT
Ligase
kerA
pLB3
pCRII
SpeI
XbaI
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TCA
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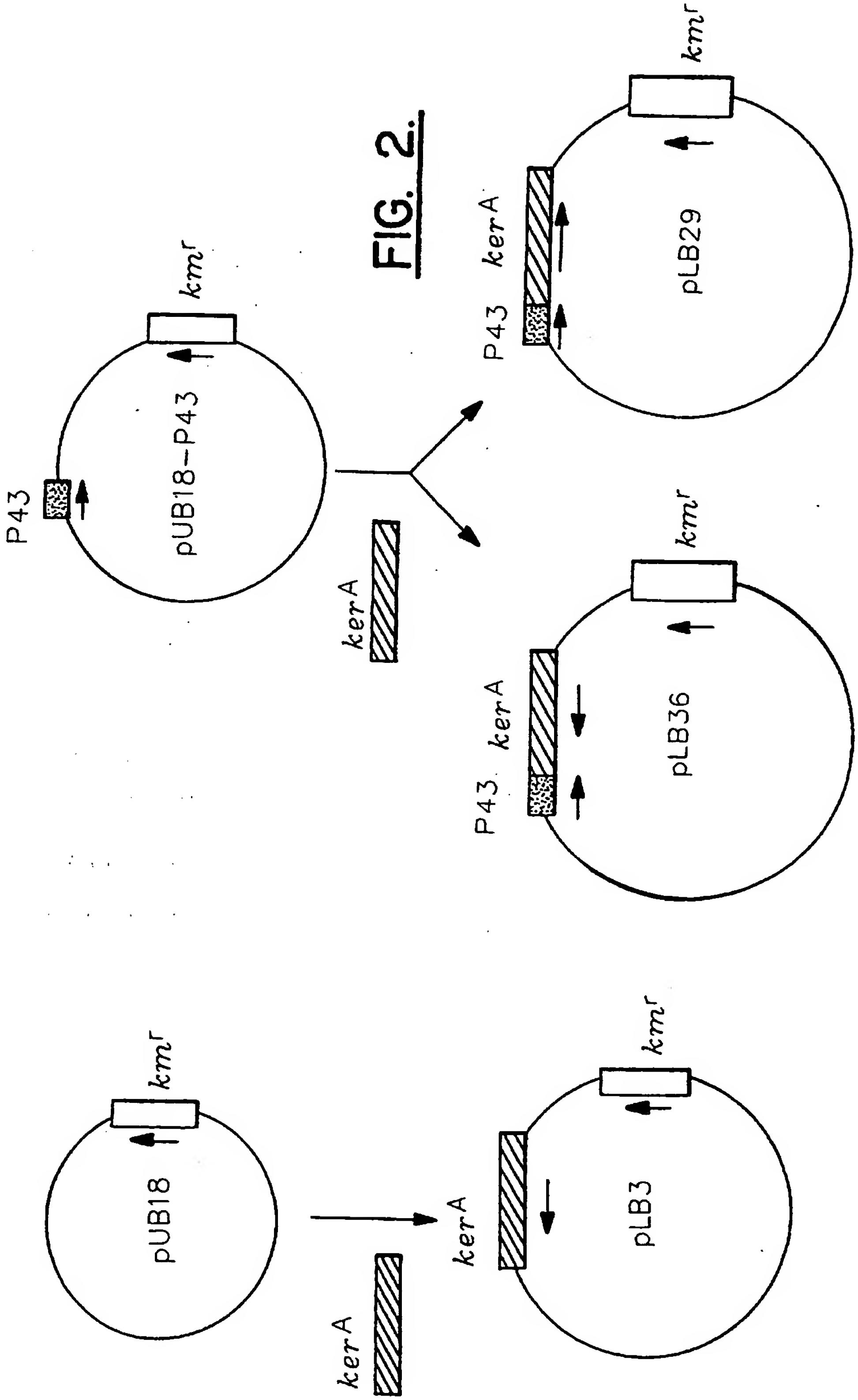
AGATC
TCT

FIG. 1.


P43
pUB18-P43
km^r
kerA
P43
kerA
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kerA
pLB36
km^r
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km^r
kerA
kerA
pLB3
km^r

FIG. 2.

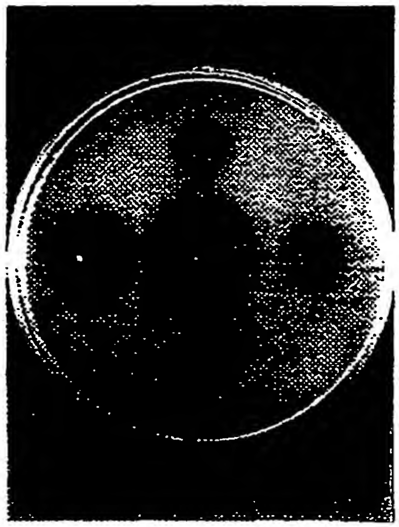

FIG.3A

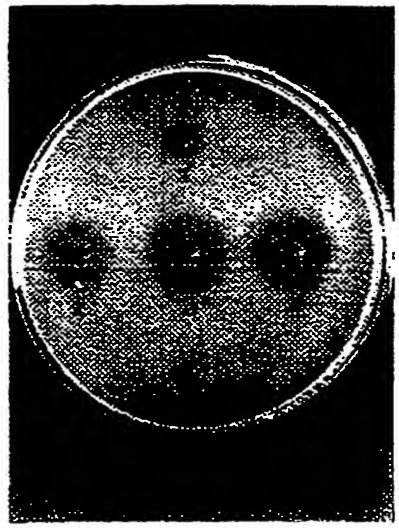

FIG.3B

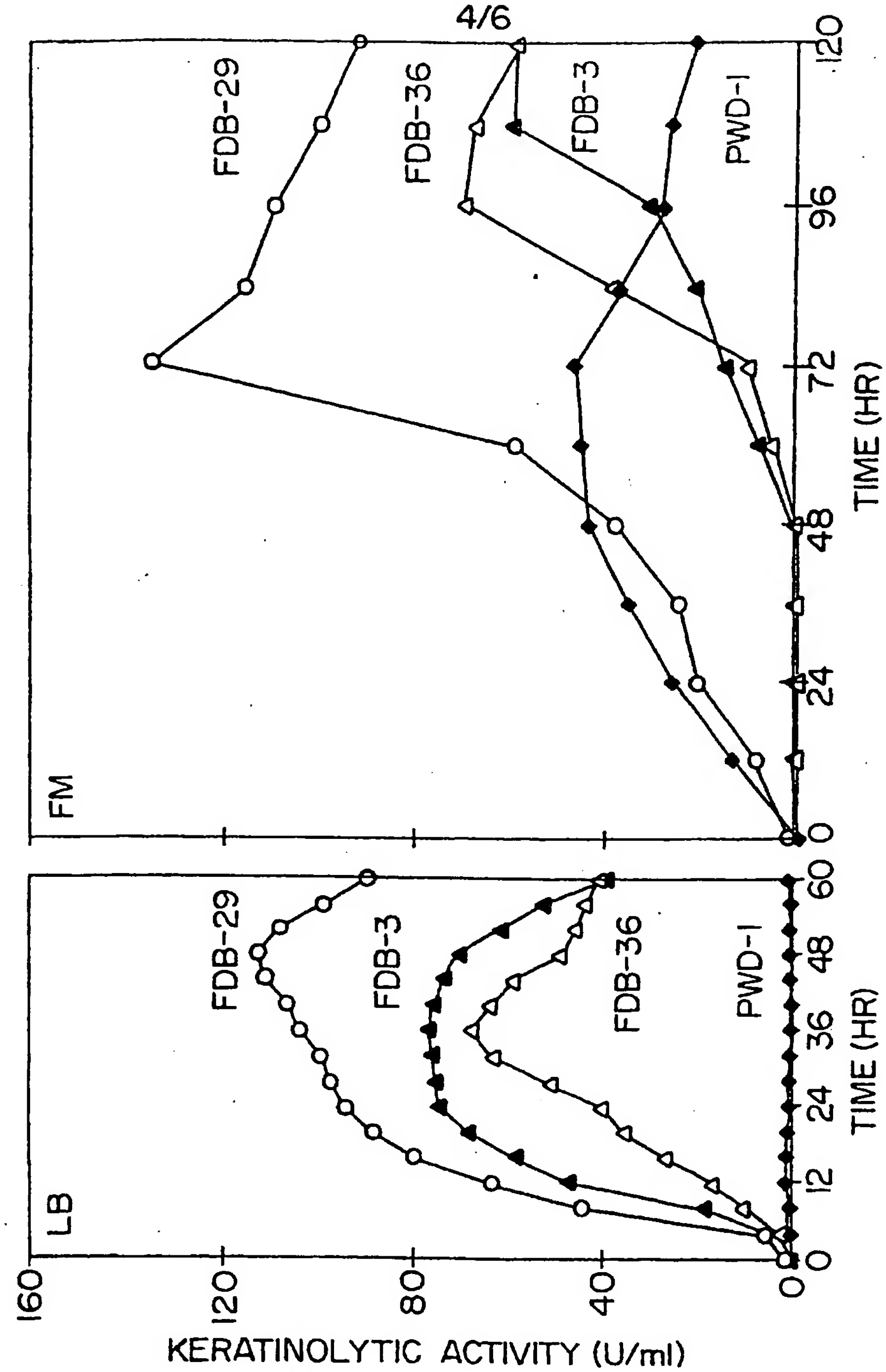
LB
FM
FDB-29
FDB-3
FDB-36
PWD-1
TIME (HR)
KERATINOLYTIC ACTIVITY (U/ml)
0
40
80
120
160
0
12
24
36
48
60
0
24
48
72
96
120

FIG. 4.

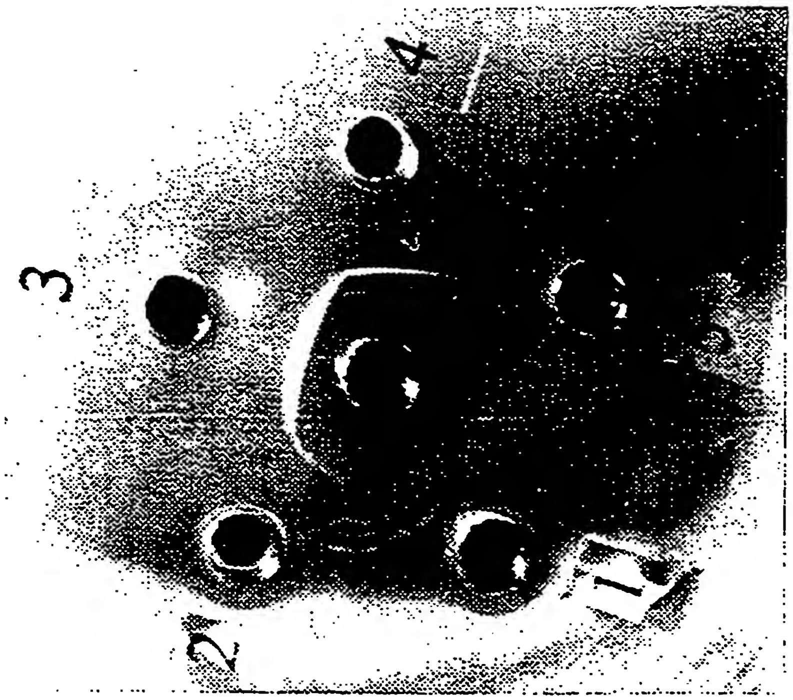

FIG. 5B.

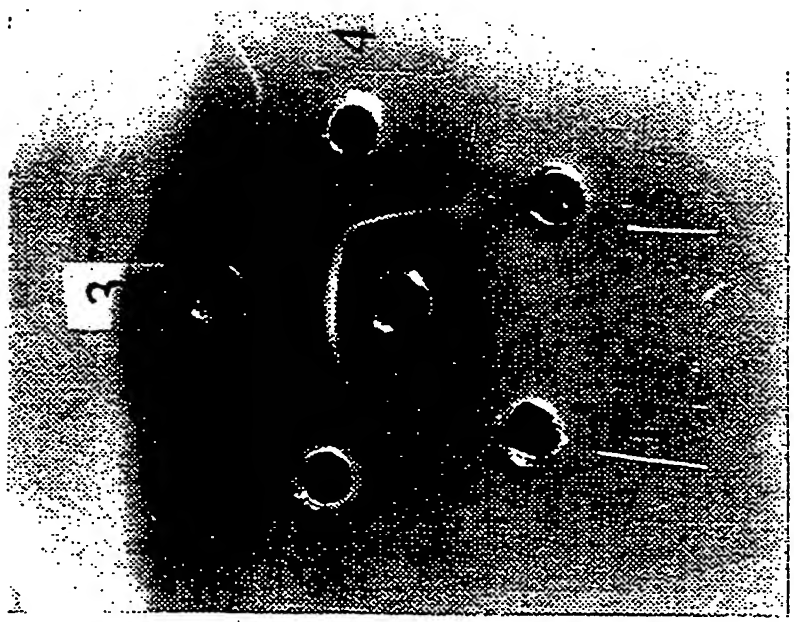

FIG. 5A.

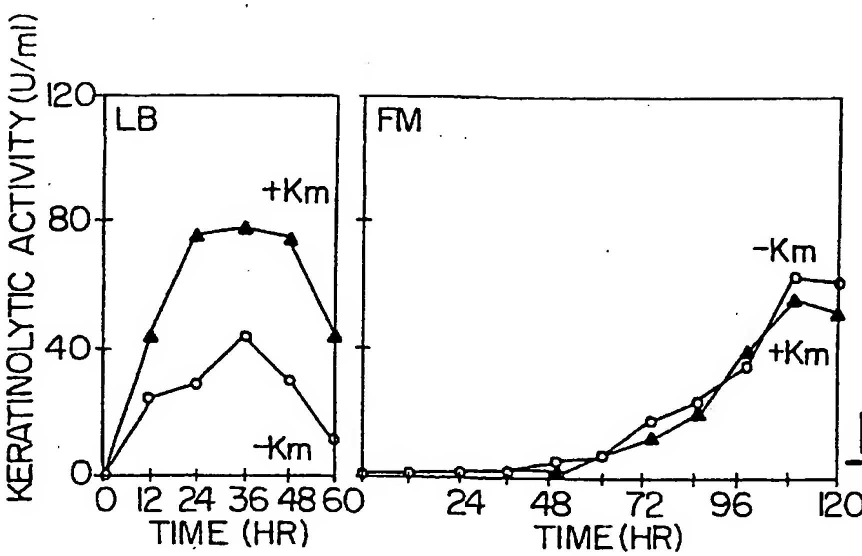

FIG. 6A.

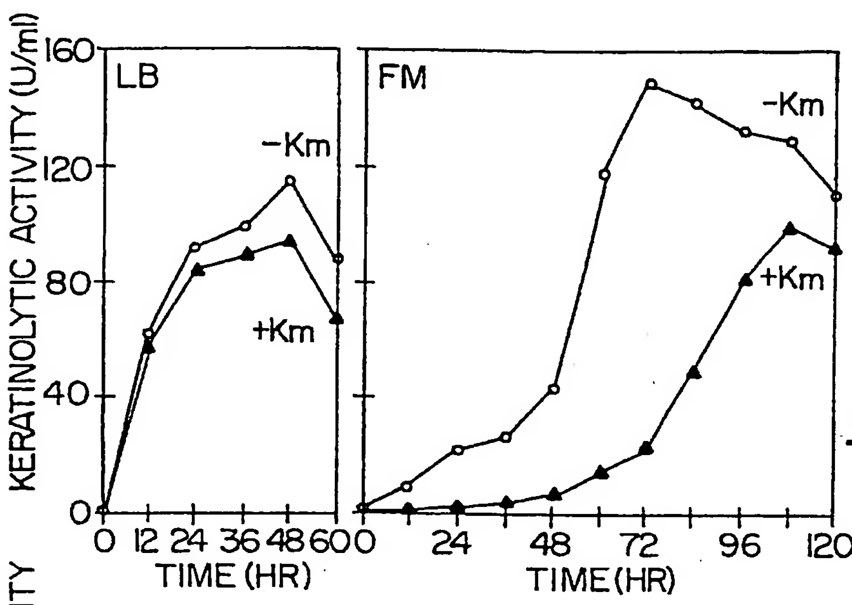

FIG. 6B.

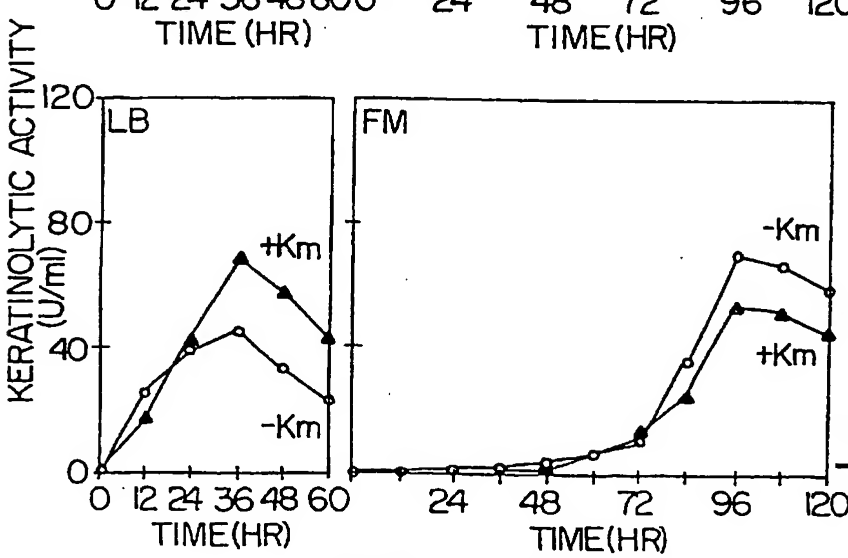

FIG. 6C.